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DISTRIBUTION OF THE ENDOSYMBIONT *NEPHROMYCES* GIARD WITHIN THE ASCIDIAN FAMILY MOLGULIDAE

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Abstract

In various anecdotal reports, nineteenth and early twentieth century authors have asserted that microbial cells, "*Nephromyces,*" are present in the renal sac of the ascidian *Molgula.* This study confirms the presence of such cells in the renal sac lumen of five *Molgula* species (*M. manhattensis, M. arenata, M. complanata, M. citrina, M. occidentalis*) and one species of the molgulid genus *Bostrichobranchus* (*B. pilularis*). This is the first report (using modern taxonomic schemes) of *Nephromyces* from a molgulid genus other than *Molgula.*

A description of the light microscope morphology of *Nephromyces* cells is also given.

Introduction

Like many structures, the "renal sac" of molgulid tunicates was named before critical demonstration of its function. Although it has often been hypothesized (or assumed) that the renal sac is an excretory organ, the biological role of this organ remains uncertain.

Recent work has focused on the morphological and chemical peculiarities of the renal sac. Most unexpectedly for an "excretory" organ, the renal sac has no openings at any stage in its development (Saffo, 1978). Consequently, it has been assumed that renal sac "waste" products are not excreted from the renal sac, but accumulated in the organ for the life of the tunicate (Das, 1948).

The renal sac lumen contains a large volume of concretions, which in composition (chiefly uric acid and calcium oxalate in *Molgula manhattensis*: Goodbody, 1957, 1965; Saffo, 1977a, b; Saffo and Lowenstam, 1978) and possible metabolic origin (Nolfi, 1970) resemble human kidney stones. Unlike kidney stones, however, these concretions show no evidence of being pathological deposits, but seem to be normal metabolic products. The chief organic component of the renal sac fluid in *M. manhattensis* has been identified as homarine (Gasteiger *et al.,* 1960; Saffo, 1976, 1977, Gaill and Lafont, 1978), a methylpyridine. It has been suggested by several authors that homarine is associated with osmoregulation, both in the renal sac of *M. manhattensis* and in the many other marine invertebrates in which the compound has been found (Gasteiger *et al.,* 1960; Lapan, 1975).

At least one feature of the renal sac—the cellular content of its lumen— has been virtually ignored in recent studies. Early papers (de Lacaze-Duthiers, 1874; Giard, 1888; Harant, 1931; Azéma, 1937) assert that fungus-like microbial cells, "*Nephromyces*" (Giard, 1888), are present in the renal sac. Despite their potential

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significance in the activities and biological role of the renal sac, these cells have received little critical attention.

A century after these early reports, even the identity of *Nephromyces* remains in doubt. Usually assuming that these cells represent a single organism, earlier workers have alternately classified *Nephromyces* as a chytridiomycete (Giard, 1888; Harant, 1931), a gregarine protozoan (de Lacaze-Duthiers, 1874), and as "a lower fungus which has no relatives, not even distant ones, among [other groups of lower fungi]" (Buchner, 1965). The cells of *Nephromyces* are so peculiar, their habitat (the renal sac) so bizarre, and published reports so scanty that several recent authors (Johnson and Sparrow, 1961; Alderman, 1976) have questioned the existence of *Nephromyces*, leading Alderman (1976) to state that "*Nephromyces* Giard must be regarded as extremely doubtful unless new evidence becomes available."

This paper confirms the presence of fungus-like cells in the renal sac lumen. A description of the light-microscope morphology of *Nephromyces* is presented, with a report of the distribution of these cells in six species of molgulid tunicates.

Materials and Methods

Collection of animals

Molgula manhattensis was collected from the following locations: San Francisco Bay, California (1972–1978, all times of year; Redwood City; Palo Alto Yacht Harbor, Berkeley Municipal Marina, Sausalito Yacht Harbor); Vineyard Sound and Cape Cod Bay, Massachusetts (summers 1977, 1979, 1980; Falmouth, Woods Hole, Vineyard Haven, Sandwich); Chesapeake Bay (September 1977; Solomons Island, Maryland; Gloucester Point, Virginia); Atlantic Coast of Florida (September 1977; Fort Pierce Inlet; Banana River); New Jersey (fall, spring, 1978–1980; Belmar Marina); and Manhattan Island, New York (October 1980; 25th Street Marina).

Molgula citrina and *Molgula complanata* were collected from Sandwich, Massachusetts (summers 1977, 1979, 1980).

Molgula arenata was dredged from Vineyard Sound, Massachusetts (summers, 1977, 1979).

Molgula occidentalis was collected from both the Atlantic (September 1977; Sebastian Inlet) and Gulf Coasts (February 1978 and December–February 1980–1981; Alligator Harbor, Panacea; Carabelle) of Florida.

Bostrichobranchus pilularis was dredged from Vineyard Sound, Massachusetts (summers 1979, 1980; 50 meters depth) and also collected from shallow water in Panacea, Florida, (February 1981; Alligator Harbor).

The *B. pilularis* and *M. occidentalis* from Alligator Harbor were supplied by the Gulf Specimen Co. (Panacea, Florida).

Examination for Nephromyces

Observations were based exclusively on living material. For small animals, the transparent renal sac was excised from the tunicate and examined, whole, with phase contrast optics at 200–400×. For larger animals, the renal sac was dissected from the tunicate, adjoining heart tissue was cut away, and the organ rinsed and blotted with filter paper to remove cells extraneous to the renal sac (*e.g.,* blood cells). Renal sac contents were then removed, placed on a slide, and examined with phase contrast or Nomarski optics at 200–1000×.

TABLE I

Presence of Nephromyces *in the renal sac of adult molgulids, 1977–1981.*

Species	Location	Number examined	Number infected
Bostrichobranchus pilularis	Gulf Coast (Florida) and Atlantic Coast (Massachusetts)	23/23	
Molgula arenata	Atlantic (Massachusetts)	23/23	
M. citrina	Atlantic (Massachusetts)	21/21	
M. complanata	Atlantic (Massachusetts)	22/22	
M. manhattensis	Pacific (California) and Atlantic (Florida to Massachusetts)	212/212	
M. occidentalis	Atlantic and Gulf Coast (Florida)	30/30	

RESULTS

In all adults of all molgulid species examined—*Molgula manhattensis, M. citrina, M. complanata, M. arenata, M. occidentalis,* and *Bostrichobranchus pilularis*—cells are present in the renal sac lumen (Table 1). These cells differ markedly in morphology from tunicate cells, and at least broadly resemble the *Nephromyces* described by earlier authors.

These *Nephromyces* forms can be divided into at least seven broad categories:

1. "Vacuolate filaments": colorless, non-septate filaments, each with a single, large, central vacuole; about 4–10 μm in width, 15 to about 100 μm in length (Fig. 2). Some possess terminal swellings; others are occasionally found in multifilament arrays (Fig. 3). The shortest vacuolate cells are, in some species (*e.g., M. citrina*), sometimes found packaged within a circular structure (Fig. 4). The cytoplasm of vacuolate filaments sometimes contains yellow refractile granular inclusions.

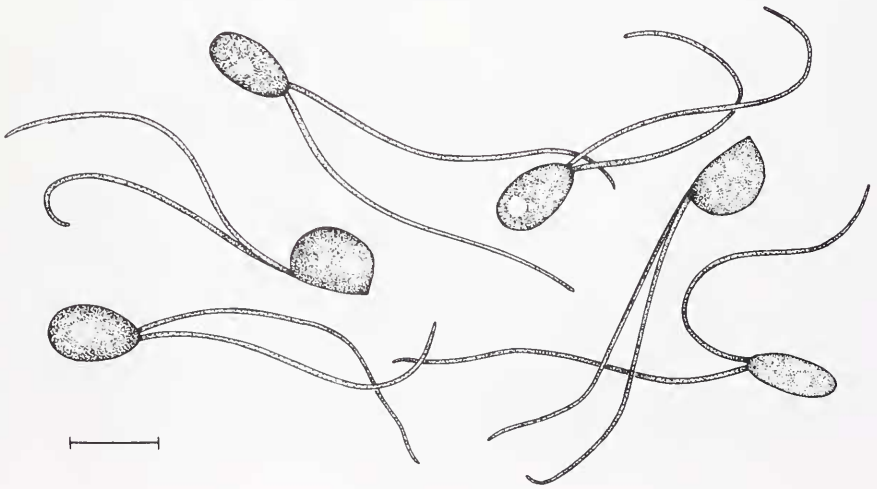
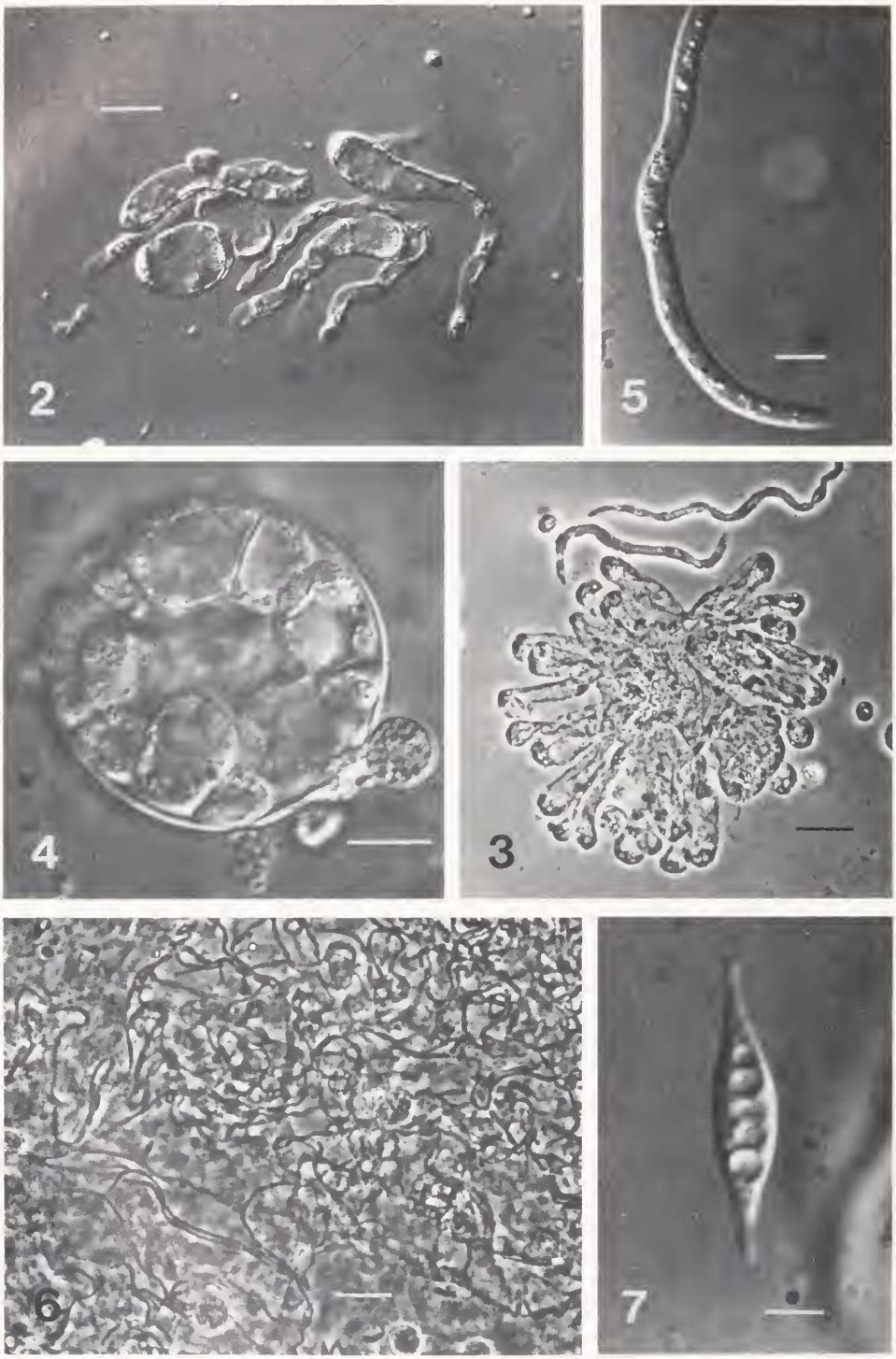


FIGURE 1. Diagrammatic representation of *Nephromyces* zoospores from *Molgula manhattensis.* Bar = 5 μm.

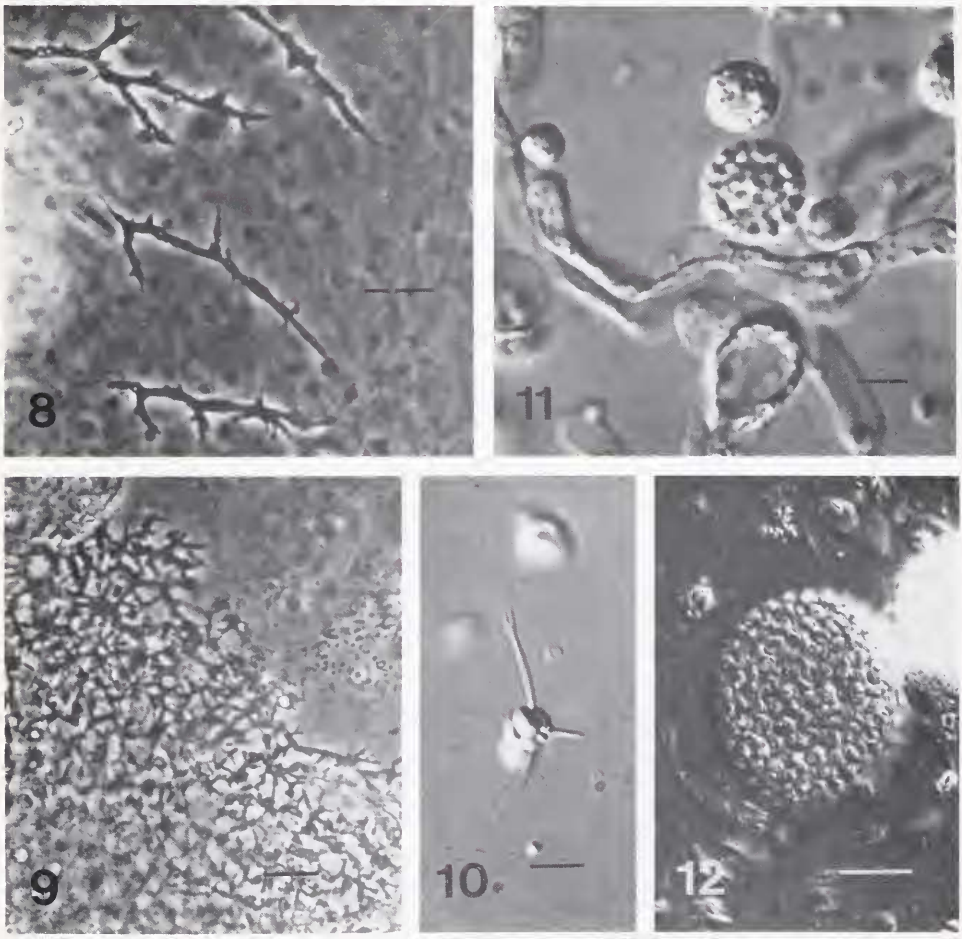


FIGURES 2–7. (2) Vacuolate filaments from *M. manhattensis*. Nomarski optics. Bar = 15 μm. (3) Multiple-armed vacuolate filament from *M. manhattensis*. Phase contrast. Bar = 20 μm. (4) Enclosed vacuolate filaments, from *M. citrina*. Nomarski optics. Bar = 10 μm. (5) Slender filament, from *M. manhattensis*. Nomarski optics. Bar = 10 μm. (6) Slender filaments, from *M. occidentalis*. Phase contrast. Bar = 30 μm. (7) Spindle-shaped filament from *M. manhattensis*. These filaments possess either no discernible vacuoles, or (as shown here) a series of small vacuoles. Nomarski optics. Bar = 10 μm.

2. "Slender filaments": colorless, non-septate filaments without large vacuoles (though occasionally with small vacuoles); 3–5 μm in width, greater than 15 μm in length (Fig. 5); occasionally, some have terminal swellings. In *M. occidentalis* (Fig. 6), these filaments can approach 400–500 μm in length. Filaments usually have rounded tips (Figs. 5, 6); sometimes, though rarely, these filaments have pointed narrow tips (Fig. 7), giving the filaments a spindle-like form.

Both vacuolate and slender filaments are "mycelial" only in the sense that they are often entangled with each other, or with other *Nephromyces* forms; they are virtually never branched, except in early growth stages (Saffo, 1982), and fusion with other filaments has so far not been observed. Occasionally, filaments with characteristics intermediate between those of vacuolate and slender filaments (slender width, but with a row of conspicuous vacuoles separated by thin bands of cytoplasm) are present, suggesting that vacuolate filaments may develop from slender filaments, or *vice versa.*

3. "Irregular filaments": colorless filaments with irregularly shaped bound-

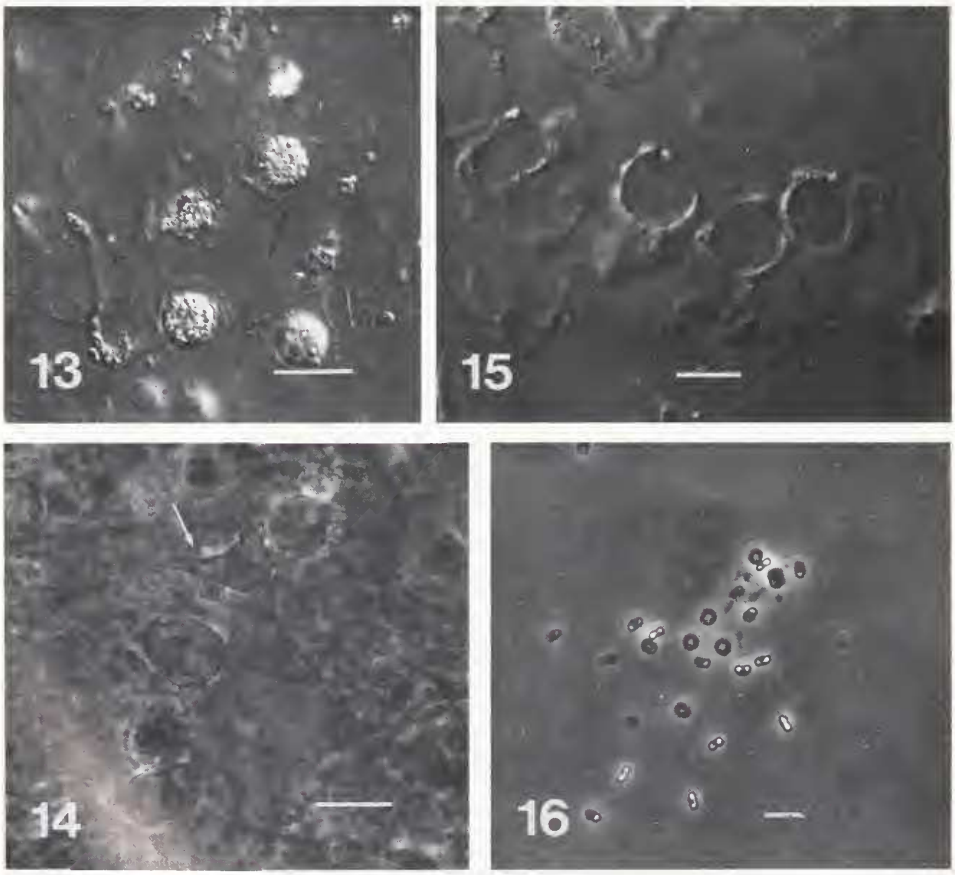


FIGURES 8–12. Irregular filaments on the inner renal sac wall of *M. manhattensis.* Phase contrast. Bar = 15 μm. (9) Network of irregular filaments from *Bostrichobranchus pilularis.* Phase contrast. *Bar* = μm. (10) A zoospore with atypically prominent apical projection, from *M. citrina.* Nomarski optics. Bar = 5 μm. (11) Sporangium (and vacuolate filaments) from *M. manhattensis.* Nomarski optics. Bar = 10 μm. (12) Atypically large sporangium from *M. complanata.* Nomarski optics. Bar = 15 μm.

aries—individually about 3–6 μm in width, typically about 40–60 μm in length (Fig. 8). Unlike other filament forms, the irregular filaments are found more often on the inner wall of the renal sac, rather than free in the renal sac lumen. They are sometimes found in network-like arrays (Fig. 9). To date, such filaments have been found only in *M. manhattensis* and *B. pilularis*.

4. Posteriorly biflagellate swarmer cells ("zoospores"): the cell is about 3–5 μm in length, with one or two refractile globules in the center of the cell. The flagella are equal in length, about 12–15 μm long (Fig. 1). In some species (*e.g., M. citrina*), zoospores occasionally possess a prominent apical projection (Fig. 10).

5. Rosette-shaped "sporangia": usually about 20 μm diameter (Fig. 11), though sometimes larger (an occasional occurrence in *M. complanata* and *M. citrina* Fig. 12). Biflagellate zoospores (above) are discharged from these. Before zoospores discharge, the sporangium is surrounded by a wall, which is apparently dissolved or torn apart during zoospore discharge. No discharge pore has been detected in the sporangium in most host species, although in *M. citrina*, sporangia are often surrounded by a heavy wall with two or three tunnel-like openings (Fig. 13); some-



FIGURES 13–16. (13) Sporangia surrounded by heavy tunneled wall (arrow), from *M. citrina*. Nomarski optics. Bar = 20 μm. (14) Sporangial wall connected to filament (arrow), from *M. citrina*. Phase contrast. Bar = 15 μm. (15) "Baskets" from *M. manhattensis*. Nomarski optics. Bar = 10 μm. (16) "Doughnut"-shaped cells from *M. manhattensis*. Phase contrast. Bar = 10 μm.

times these walled structures seem to be connected to filaments (Fig. 14). Yellowish spherical granular cells, about 20 μm in diameter, are also present; their size and form suggest that they are uncleaved sporangia.

6. "Baskets": irregularly shaped structures (Fig. 15), open at one end (prominent rings in Fig. 15), and virtually devoid of cytoplasm; about 25–50 μm in diameter. Though it is not clear whether these structures are even living cells, they may represent remnants of some *Nephromyces* cell type. Certainly they are associated only with *Nephromyces*. I have never seen them anywhere in *Molgula* other than in the renal sac, and they do not appear in experimentally *Nephromyces*-free *Molgula* (Saffo and Davis, 1982).

7. "Doughnuts": heavy-walled flattened circular cells, about 3–5 μm in frontal diameter (Fig. 16). The behavior of these cells will be described more fully elsewhere (Saffo, unpublished).

Except for the irregular filaments (3, above), all these cell types were found in the six molgulid species examined. All these cells were usually (but not invariably) found in each adult of each species, though relative numbers of each cell type often varied from individual to individual. In contrast to Giard (1888) and Harant (1931), I have seen no qualitative differences in cell-type distribution with season, at least in adult *M. manhattensis* (the only species sampled at all times of year).

Discussion

Although these observations are broadly similar to those of earlier authors, their descriptions do differ from mine—and from each other—in many details, where they provide these at all.

De Lacaze-Duthiers (1874) stated that filaments (reminiscent of types 1 and 2 above), were "almost always present" in *M. tubulosa* (=*M. occulta*: Berrill, 1950), but he saw no other forms.

Giard found "*Nephromyces molgularum*" in *M. socialis* (=*M. manhattensis*: Berrill, 1950); "*N. sorokini*" in *Lithonephrya eugyranda* (= *M. complanata*: van Name, 1945) and "*N. roscovitanus*" in *Anurella roscovitana* (=*M. occulta*: Berrill, 1950). Of these, he described only *Nephromyces molgularum* in any detail and provided no illustrations. This species, according to Giard, possesses a mycelium of delicate, entangled filaments, some of which have terminal swellings. Zoosporangia of "very diverse form" liberate tiny, uniflagellate zoospores with a refringent granule near the base of the flagellum. In autumn, zygospores are formed where "four or five" mycelial filaments fuse; in winter, filaments germinate from these zygospores. Vacuolated filaments are present all year long.

Harant (1931) found filaments of various lengths, with and without vacuoles, in the molgulid *Ctenicella appendiculata* (=*Molgula appendiculata*: Buchner, 1930). Harant also described filaments which bear spiny-surfaced "resistant spores," which he considered markedly different from the zygospores of Giard. He reported that cylindrical, vacuolated cells develop into zoosporangia; these release "zoospores" with a lipid globule and single, long, apical flagellum. These "zoospores" (belying the asexual implications of their name) fuse with each other as gametes.

Of all the earlier reports of *Nephromyces*, Buchner's (1930, 1965) descriptions of *Nephromyces* from *M. impura* most nearly resemble those presented here—at least in description of the morphology of *Nephromyces* cells, if not in interpretation of their developmental roles. Buchner (1965) also noted that he found "more or

less similar forms (of fungi) in a long series of molgulids, preserved in alcohol, from all parts of the earth."

At the light microscope level, each of the cell types described here, as in earlier papers, bears a superficial resemblance to very different kinds of microorganisms.

The non-septate filaments (types 1 and 2) resemble phycomycetous fungi. The irregularly-bounded filaments seem more similar to slime molds (and associated mycetozoans) than to fungi. The sporangia strongly resemble those of thraustochytrids (*e.g.,* Goldstein, 1963) in behavior and in light microscope structure; but the swarmers that are discharged from them are markedly different from those of thraustochytrids, even at the light microscope level. The swarmer cells do resemble chytrid zoospores in size, in the presence of refractile globules, and in their posterior flagellation. However, chytrid zoospores are typically uniflagellate rather than biflagellate; in fact, there is no flagellate protistan group with posteriorly biflagellate zoospores (Saffo, 1981). At the light microscope level, the apical projection in some *Nephromyces* swarmers (Fig. 10) broadly resembles a short or rudimentary variant of the haptonema borne on flagellated cells of the algal haptophytes (Prymnesiophyceae), except that the *Nephromyces* projection does not appear to be motile. Finally, in their small size and simplicity of surface (light microscope) morphology, the doughnut-shaped cells resemble prokaryote cells more than they do eukaryotes.

The appearance of *Nephromyces* raises interesting phylogenetic questions (Saffo, 1981), to which ultrastructural data will contribute essential portions of the answers. Meanwhile, two basic questions must be addressed.

If *Nephromyces* cells resemble so many different kinds of organisms, is *Nephromyces* one kind of organism, or a collection of several kinds of organisms? Though these observations do not prove that *Nephromyces* is (within each host species) a single organism, they are consistent with this hypothesis. In the six molgulid species studied, the same categories of *Nephromyces* cells appear repeatedly, with only slight variations from host species to host species. If *Nephromyces* were merely a group of unrelated organisms inhabiting the renal sac, it would be difficult to imagine the same (or similar) community persisting in species after species of molgulids, despite wide differences in (1) morphology, (2) habitat (floats and pilings, *M. manhattensis* and *M. citrina*; sandy bottom, *M. arenata*; intertidal rocks, *M. complanata*; mud and sandy mud, *B. pilularis*), and (3) developmental pattern (oviparous, *M. manhattensis, M. occidentalis, M. arenata*; viviparous, *M. citrina, M. complanata*; oviparous with direct development, *B. pilularis*) of the host species in question. More substantial developmental evidence in support of this hypothesis is presented elsewhere (Saffo, 1981, 1982). The only occasional presence of irregular filaments is not inconsistent with this hypothesis, since irregular filaments are almost certainly an early or alternate developmental stage of the slender filaments (Saffo, unpublished).

Slight variations in *Nephromyces* are found in different host species. Such differences persisted even in the single case where two molgulids (*M. manhattensis* and *M. citrina*) cohabited the same area (Sandwich Marina). Either different host species do possess different *Nephromyces* species, as Giard suggested (1888), or the renal sacs in different host species are sufficiently different habitats to induce developmental differences in a single *Nephromyces* species.

Is *Nephromyces* an organism at all, or merely molgulid cells? If one accepts the older reports, it can be concluded that *Nephromyces* is present in molgulids not only from the western Atlantic and eastern Pacific, but also from the eastern Atlantic and Mediterranean, and in three molgulid species in addition to the six enumerated in this paper. At the very least, the distribution of *Nephromyces* within

the Molgulidae is taxonomically and geographically widespread. The presence of *Nephromyces* in *Bostrichobranchus pilularis* is particularly striking, as it is the first report (using current taxonomic schemes) of *Nephromyces* in a molgulid other than the genus *Molgula*. Indeed, these results suggest that its distribution may well be universal among adult molgulids. I have not found any adult molgulid specimen that does not contain *Nephromyces* in its renal sac.

The possibly universal distribution of *Nephromyces* leads one to question whether *Nephromyces* is a microorganism or merely a collection of molgulid cells. At the light microscope level, *Nephromyces* cells do not look like tunicate cells. A subsequent paper (Saffo and Davis, 1982) presents critical evidence that *Nephromyces* is, indeed, something foreign to its host.

If *Nephromyces* is not merely a collection of molgulid cells, its universal distribution among the molgulid species studied, and, by implication, widespread distribution throughout the Molgulidae make it difficult to imagine that *Nephromyces* has grossly pathologic effects on its hosts. Though it has not been demonstrated that the association between molgulids and *Nephromyces* is mutualistic, it does seem to be an intimately coevolved association. Certainly, investigation of this association appears to be essential for understanding of both the role of the renal sac, and, more generally, of the evolution and ecology of the common, but poorly understood Molgulidae.

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